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Award Number: DAMD17-98-1-8190

TITLE: Structural Characterization of a Novel HER2/neu Binding
Ets Factor, ESX

PRINCIPAL INVESTIGATOR: Christa Nunes, M.S.

C. Benz, M.D.

CONTRACTING ORGANIZATION: University of California
San Francisco, California 94143-0962

REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGEForm Approved
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 99 - 30 Jun 00)	
4. TITLE AND SUBTITLE Structural Characterization of a Novel HER2/neu Binding Ets Factor, ESX			5. FUNDING NUMBERS DAMD17-98-1-8190	
6. AUTHOR(S) Christa Nunes, M.S. C. Benz, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California San Francisco, California 94143-0962 E-MAIL: cnunes@socrates.berkeley.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Understanding the structural basis of protein-nucleic interactions involved in breast cancer cell proliferation would prove beneficial to the rational design of therapeutic strategies aimed at reducing proliferation. We have focused on two proteins, ESX and Fmu, which serve as models for understanding the structural role of protein-nucleic acid interactions in the growth control of breast cancer. ESX, an Ets family transcription factor, binds to the Ets response element of the HER2 promoter and upregulates HER2 transcription. Fmu, a cytosine methyltransferase that modifies rRNA, is the bacterial homolog of human nucleolar p120, an enzyme overexpressed in human breast tumors and likely critical to the proliferative phenotype. The research goal is to determine these protein structures by x-ray crystallography and define the specific contacts with their binding targets to enable the design of small molecule modulators of proliferative function. As crystals of ESX have not been obtained, we have focused on Fmu structure determination. To date, we have obtained 1.65 Å native data and 2.25 Å MAD phases, and have built 65% of the protein model into the experimental maps. Structure determination of Fmu will provide insight into RNA binding and modification, and present a novel target for breast cancer intervention				
14. SUBJECT TERMS Breast Cancer, X-ray crystallography, proliferation, ESX, Fmu				15. NUMBER OF PAGES 11
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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INTRODUCTION:

ESX is an Ets family transcription factor that binds to the Ets response element of the HER2/*neu* promoter (GAGGAA) where it causes up-regulation of HER2 transcription. Overexpression of the HER2/*neu* proto-oncogene in breast cancer occurs in approximately 20% of cases and is related to poor patient prognosis. In our initial proposal, we proposed to express, purify, crystallize and determine the 3-dimensional structure of ESX by x-ray crystallography. Elucidation of the ESX structure would be beneficial for the development of novel therapeutics aimed at blocking ESX transactivating function. Due to the difficulties in obtaining crystals of ESX, our focus has shifted to structural studies of Fmu, a novel protein that will provide insight into the role of an rRNA methyltransferase implicated in breast cancer. Fmu, a 429 amino acid RNA m⁵C methyltransferase from *E. coli*, methylates the 5-carbon of C967 of 16S rRNA. It is composed of a 270 amino acid C-terminal methyltransferase domain, and a 159 amino acid N-terminal region that is proposed to be an RNA binding domain. Fmu is the bacterial homolog of yeast Nop2 and human p120, which all share a conserved methyltransferase domain that is flanked on each end by variable regions.⁽¹⁾ Nucleolar human p120 is a proliferation associated protein which has been found to be over-expressed in tumor cells. Most human malignant tumors contain much higher levels of protein p120 than normal resting cells⁽²⁾, and expression of p120 is used as a prognostic indicator. Furthermore, increased expression has been correlated with early recurrence and shorter survival in breast cancer patients.⁽³⁾ p120 has also been shown to be a marker of aggressiveness in lung, prostate, colorectal and oral carcinoma.^(4,5,6,7) Recent work has shown that the inhibition of p120 in tumor cell lines (by either ribozyme vectors or anti-p120 antibody) results in decreased cell growth^(8,9), and these results suggest p120 is an attractive target for therapeutic intervention. As p120 is homologous to Fmu, structural information obtained for Fmu would be directly applicable to p120. Here we propose to express, purify, crystallize and determine the structure of the multi-domain RNA modifying enzyme Fmu by x-ray crystallography. The ultimate goal of this research is to use structural analysis to define inter-domain interactions of Fmu or specific contacts between Fmu and rRNA, which can later be targeted for the design of small molecule modulators of proliferative function. The following is a summary of our progress toward these goals.

BODY:**Technical Objective 1: Expression and purification of protein domains for structural analysis.**

The cloning of the *fmu* gene and production of the wild-type protein has been previously described.⁽¹⁰⁾ Selenomethionine-labelled Fmu used for MAD phasing experiments was produced from 4 liters of BL21(DE3)pLysS cells as per the method of van Duyne.⁽¹¹⁾ The purification of SeMet-labelled protein is as follows: 4 liters of cells were pelleted and resuspended in 50 ml Buffer A (20mM Tris HCl (pH 7.5), 50mM NaCl, 1mM MgCl₂, 0.5mM DTT, 10%glycerol), and the resuspended cells were lysed by sonication. The lysed cells were centrifuged for 20 minutes at 12,000 rpm to remove the cellular debris. The supernatant was decanted into a new tube and loaded onto a pre-equilibrated 30 ml Q-sepharose column (Buffer A). The column was washed with ~10 column volumes of Buffer A, and the protein eluted with a gradient of 0 to 0.5 M KCl in buffer A. The fractions were pooled after checking by SDS-PAGE and dialyzed overnight against Buffer B (10mM KPi (pH 6.8), 0.5mM EDTA, 0.5mM DTT 10%glycerol). The dialyzed sample was loaded onto a HAP column pre-equilibrated with buffer B and eluted with a linear gradient of 10 to 500mM KPi (pH 6.8) in buffer B. The pooled fractions were concentrated to about 6 mg/ml.

Technical Objective 2: Crystallization of protein.

Crystal screens were initiated using Fmu purified as described above. Drops were placed on siliconized cover slips and inverted over 1 ml of well buffer. Well buffers were obtained from commercially available sparse matrices containing greater than 10 distinct precipitants combined with different salts and additives (Hampton Research). No crystals were seen in the initial trays, however a shiny precipitate was observed in drops with PEG as the precipitant. Following this result, a set of PEG conditions were made (5-30% PEG 400-PEG 8000) over a pH range of 5.5 to 8.5 (0.1M buffer) and more crystallization trials were preformed. Small crystals were observed in drops with 15% PEG 4000, pH 8.5. A finer grid screen of PEG solutions was made (12-18% PEG 4000, 0.1M Tris pH 8.0-8.6) and further crystallization trials produced crystals suitable for diffraction.

Technical Objective 3: Single crystal diffraction of protein

The crystals obtained as described above were tested for diffraction on a home source, a Raxis IV rotating anode X-ray generator equipped with a high speed area detector. Since cryoprotection can significantly increase the useful diffraction lifetime of protein crystals, potential cryosolvents were screened for their ability to allow flash freezing of Fmu crystals in liquid nitrogen. Mineral Oil was determined to be the best cryoprotectant for these crystals. The crystals diffracted to approximately 3.0 Å in space group P2₁ (a=59.48, b=48.64, c=86.65, α = 90.0, β =108.59, γ =90.0). Following initial screening on the home source, crystals were taken to a synchrotron at Stanford Synchrotron Radiation Laboratory. A dataset was collected on a native crystal that diffracted to 1.65 Å.

Technical Objective 4: Structural solution and interpretation

In addition to high quality diffraction, structural solution requires accurate phasing information. In order to obtain phasing information, selenomethionine Fmu protein was purified as described above. SeMet Fmu crystallized in conditions identical to those for the native crystals. A three wavelength MAD (Multi-wavelength Anomalous Dispersion) experiment was collected at the Advanced Light Source at Lawrence Berkeley Laboratories. The selenomethionine Fmu crystal diffracted to 2.25 Å in space group $P2_1$ ($a=87.53$, $b=48.56$, $c=114.83$, $\alpha=90.0$, $\beta=107.79$, $\gamma=90.0$). Using this data and the crystallographic software program CNS, 8 selenomethionine sites (there are 9 total per protein) were found. Starting phases were calculated using these 8 sites and the software SHARP. Non-crystallographic symmetry averaging was performed since there were 2 molecules in the asymmetric unit, and initial maps were calculated. The molecule was traced into the electron density with iterative rounds of building and map calculation. This solution was used to fit the 1.65 Å data using molecular replacement and we are now building into that unit cell. To date, we have 65% of the protein built into the experimental maps and we are still fitting atoms into the electron density.

Interpretation of the structure will follow the completion of building.

KEY RESEARCH ACCOMPLISHMENTS:

- Screened crystallization conditions for Fmu
- Obtained crystals of Fmu
- Expressed and purified selenomethionine-labelled Fmu
- Identified a cryoprotectant condition and collected diffraction data for Fmu
- Obtained phase information for Fmu data and calculated initial maps
- Built partial model of Fmu that was used in Molecular Replacement to fit the 1.65 Å data
- Currently building the remaining Fmu model into the electron density maps

REPORTABLE OUTCOMES:

None to date.

CONCLUSIONS:

As mentioned in the introduction, the focus of the proposal has shifted since crystals were not obtained for ESX. The progress made on Fmu, however, meets the proposed timeline in the original grant. Fmu has been expressed, purified, and crystallized, with diffraction data collected to a maximum resolution of 1.65 Å. Additionally, selenomethionine-labelled Fmu has been purified, crystallized and used for structural determination by MAD phasing methods. The resulting electron density maps are of excellent quality, and two-thirds of the molecule has been built into the density. The refined protein structure will define the structural basis for cofactor (S-Adenosylmethionine) and RNA binding, and provide insights into the m⁵C methyltransferase mechanism of Fmu. Such information will provide a starting point for identifying regions that may be suitable for inhibitor design.

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